



The proximity between helix I and helix XI in the melibiose carrier of *Escherichia coli* as determined by cross-linking

Ping Z. Ding, T. Hastings Wilson *

Department of Cell Biology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115, USA

Received 23 April 2001; received in revised form 28 June 2001; accepted 3 July 2001

Abstract

The melibiose carrier of *Escherichia coli* is a transmembrane protein that comprises 12 transmembrane helices connected by periplasmic and cytoplasmic loops, with both the N- and C-termini located on the cytoplasmic side. Our previous studies of second-site revertants suggested proximity between several helices, including helices XI and I. In this study, we constructed six double cysteine mutants, each having one cysteine in helix I and the other in helix XI: three mutants, K18C/S380C, D19C/S380C, and F20C/S380C, have their cysteine pairs near the cytoplasmic side of the carrier, and the other three, T34C/G395C, D35C/G395C, and V36C/G395C, have their cysteine pairs near the periplasmic side. In the absence of substrate, disulfide formations catalyzed by iodine and copper-(1,10-phenanthroline)₃ indicate that helix I and helix XI are in immediate proximity to each other on the periplasmic side but not on the cytoplasmic side, as shown by protease cleavage analyses. We infer that the two helices are tilted with respect to each other, with the periplasmic sides in close proximity. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Proximity; Transmembrane helix; Cross-linking

1. Introduction

The melibiose carrier of *Escherichia coli* is a cation-substrate cotransport protein unique in its ability to utilize H⁺, Li⁺ or Na⁺ as the coupling cation [1–6]. The gene for the melibiose carrier (melB) was cloned and sequenced [7,8]. The primary sequence of the protein predicts high hydrophobicity with 70% of the residues nonpolar, and a molecular mass of 52 kDa [8]. Based on hydropathy analysis

combined with information from melB–phoA fusions [8–11] and protease cleavage [12], a secondary structure has been predicted of 12 transmembrane α -helices connected by hydrophilic loops with both N- and C-termini on the cytoplasmic side of the membrane (Fig. 1).

Na⁺ and Li⁺ increase the carrier's affinity for melibiose in membrane vesicles, and the cations compete for the same binding site [13,14]. A 1:1 stoichiometry for Na⁺/sugar cotransport has been demonstrated [14,15]. Cation recognition has been investigated by site-directed mutagenesis of negatively charge residues in helix I (D19, D35), helix II (D55, D59) and helix IV (D124) to neutral residues [16–20]. While sugar binding in those mutants remains comparable to that of the wild-type carrier

* Corresponding author. Fax: +1-617-432-1144.

E-mail address: thomas_wilson@hms.harvard.edu (T.H. Wilson).

in the absence of Na^+ , the binding is not stimulated by Na^+ . The results have led to a proposed network for cation binding/recognition comprising D19, D55, D59 and D124 [16–20]. Our investigation on the modification of charged residues in the transmembrane domain indicated high degree of stringency on their native structures (unpublished observations), in striking contrast to lactose permease.

So far, cysteine scanning mutagenesis of transmembrane helices and the subsequent PCMBs inhibition of the cysteine mutants, and the screening of second-site revertants from inactive mutants have been our major thrust to gain structural information of the carrier protein [20–23]. From these studies we mapped out surfaces on the helices facing the aqueous channel, residues important for transport, and proposed proximities between the helices under study. Salt bridges between oppositely charged residues have been suggested between R52 and D55 (both in helix II) and D19 (helix I) [24]; D59 (helix II) and K377 (helix XI) [25,26]. They presumably bring the helices in close proximity to one another.

Cross-linking between helices would provide direct evidence for their proximity. Based on the suggestions from second-site revertants studies, we focus on helix I and helix XI in this communication, with an intent to demonstrate their proximity by showing the site-directed thiol cross-linking between them from both ends of the helices.

2. Materials and methods

2.1. Reagents

Melibiose (6-*O*- α -D-galactopyranosyl-D-glucopyranoside), iodine, cupric sulfate, and 1,10-phenanthroline monohydrate were from Sigma. *N,N'*-1,2-phenylenedimaleimide (o-PDM) and *N,N'*-1,4-phenylenedimaleimide (p-PDM) were from Aldrich. [^3H]Melibiose was a generous gift from Dr. Gerald Leblanc of the Department de Biologie Cellulaire et Moléculaire du CEA, Villefranche-sur-mer, France. [α - ^{33}P]dATP was from Andotek. Bacteriological media were from Difco. [^{35}S]Protein A was from Amersham. Endoproteinase Glu-C and α_2 -macroglobulin were from Roche. All other chemicals were reagent grade.

2.2. Bacterial strains and plasmids

Escherichia coli DW1(lacI $^+$ Δ ZYmel Δ AB) [27] was used as the host strain for plasmids. The gene for the cysteine-less carrier was inserted in vector pKK223-3 (Pharmacia Biotech) as described previously [28]. This plasmid was used as the base for site-directed mutagenesis. The DW1 cells were used for transport and carrier protein level determination.

2.3. Site-directed mutagenesis DNA sequencing

The Quick Change Double-Stranded Site-Directed Mutagenesis kit (Stratagene) was used to replace the target amino acids with cysteine one at a time. The mutagenesis primers ranged from 40 to 45 nucleotides long were made at Biopolymers Laboratory at Harvard Medical School. Double-stranded plasmid DNA was isolated with the Qiaprep Spin Miniprep kit (Qiagen) from 2-ml cultures, checked on 1% agarose gel to ensure quantity and molecular mass, and sequenced using [α - ^{33}P]dATP and primers that anneal at approximately 200 bp intervals with Amplicycle Sequencing kit (Perkin Elmer).

2.4. Immunodetection and quantitation of the melibiose carrier

The amount of the carrier protein in each strain was determined as described by Lolkema et al. [29]. In summary, a known quantity of cells was lysed with NaOH/sodium dodecyl sulfate (SDS) and neutralized on nitrocellulose filters. The filters were incubated with BSA to block nonspecific bindings, followed by incubation with a polyclonal antibody directed at the C-terminal 10 residues of the carrier [30]. [^{35}S]Protein A was used to label the bound antibody and the amount of the label was quantified by liquid scintillation counting. To correct for the nonspecific adsorption, values from strain DW1/pKK223-3 (without melB) were used as background control in each determination. The values for the double cysteine mutants are presented as a percentage of the wild-type level.

2.5. Assays for melibiose transport by intact cells

The plasmid-containing strains grown from over-

night culture (0.2 ml) was transferred to 10 ml LB medium containing 100 µg/ml ampicillin until reaching log phase (OD 200 on Klett spectrophotometer). The cells were harvested and washed twice with 100 mM MOPS adjusted to pH 7 with Tris base and containing 10 mM NaCl and 0.5 mM MgSO₄.

The cells were resuspended in 5 ml of the same buffer. [³H]Melibiose (1 µCi/ml, 1 mM) was added to an aliquot of the cell suspension (which was brought to room temperature) to a final concentration of 0.1 mM. After incubation for 10 min at room temperature a 200-µl sample was filtered through a 0.65-µm cellulose nitrite filter (Sartorius). The filter was immediately washed with 4 ml of buffer and counted by liquid scintillation. The value of background control DW1/pKK223-3 was subtracted from that of each mutant.

2.6. Membrane vesicles preparation

Forty ml plasmid-containing bacteria were grown to log phase, harvested, washed and resuspended in 5 ml 100 mM MOPS containing 250 mM sucrose. Inside-out vesicles were prepared by passing the cell suspension through a French Press (SLM-Aminco) at 8000 psi [31]. Unbroken cells were removed by centrifugation at 12000×*g* for 15 min. Membrane vesicles were stored in 100 mM MOPS with 250 mM sucrose –20°C before cross-linking analysis.

2.7. Cross-linking and protease cleavage analysis

An aliquot of 100-µl membrane vesicles was cooled at 4°C for 10 min before cross-linking. Then a very small volume (a few µl) of concentrated I₂ (dissolved in ethanol) or Cu(1,10-phenanthroline)₃ (by mixing freshly prepared aqueous solution of CuSO₄ and DMF solution of 1,10-phenanthroline monohydrate of appropriate concentrations) or PDM (dissolved in ethanol) was added to the membrane vesicle solution to a final concentration of 0.5 mM. The reaction was allowed to proceed for 30 min at 4°C. The mixture was then depleted of the cross-linking reagents before enzyme cleavage by either ultracentrifugation at 100 000×*g* to separate membrane pellets from the supernatant or dialysis using Slide-A-Lyzer cassette (from Pierce) in 100 mM MOPS with 250 mM sucrose. One µl endoproteinase

GluC was added to 20 µl of the above membrane vesicle solution (medium was 50 mM ammonium bicarbonate (pH 7.8) in addition to 100 mM MOPS/250 mM sucrose) to a final concentration of 50 µg/ml. The reaction was allowed to proceed for 60 min at room temperature and quenched with α₂-macroglobulin to a final concentration of 2 mg/ml before SDS–polyacrylamide gel electrophoresis (SDS–PAGE) analysis. The enzyme has two cleavage sites on the carrier protein [12] (Fig. 1). The enzymatic cleavage products and the controls (without the enzyme) were analysed on a 18% SDS–PAGE electrophoresis followed by transferring onto PVDF membranes. Anti-melB C-terminus antibody was used to label the protein bands. A second antibody, horseradish peroxidase linked anti-rabbit IgG, was applied to visualize the melB antibody labeled bands through luminol/peroxide reaction (New England BioLabs).

3. Results

When constructing the double cysteine mutants for thiol cross-linking between different helices on both the cytoplasmic and periplasmic sides we took into account the following factors: first, both cysteines should be facing the aqueous channel so that cross-linking can possibly occur. This was judged from the helical wheel plots of both helices (Fig. 2). Second, we considered the protein expression levels and the transport activities, of the single cysteine mutants in the pair. The properties of the relevant single cysteine mutants are in Table 1.

3.1. Characteristics of the double cysteine mutants

Six double cysteine mutants were constructed. Three of them have cysteine pairs near the periplasmic side and the other three near the cytoplasmic side (Fig. 1 and Table 2). Two of the double mutants, K18C/S380C and T34C/G395C, have only 4–5% of the normal protein expression level. Only F20C/S380C and V36C/G395C have transport activities. We do not see any obvious correlation between the single and double cysteine mutants with respect to their protein expression levels. However, the transport activity of the double mutant seems to require that both the single mutants have good activities.

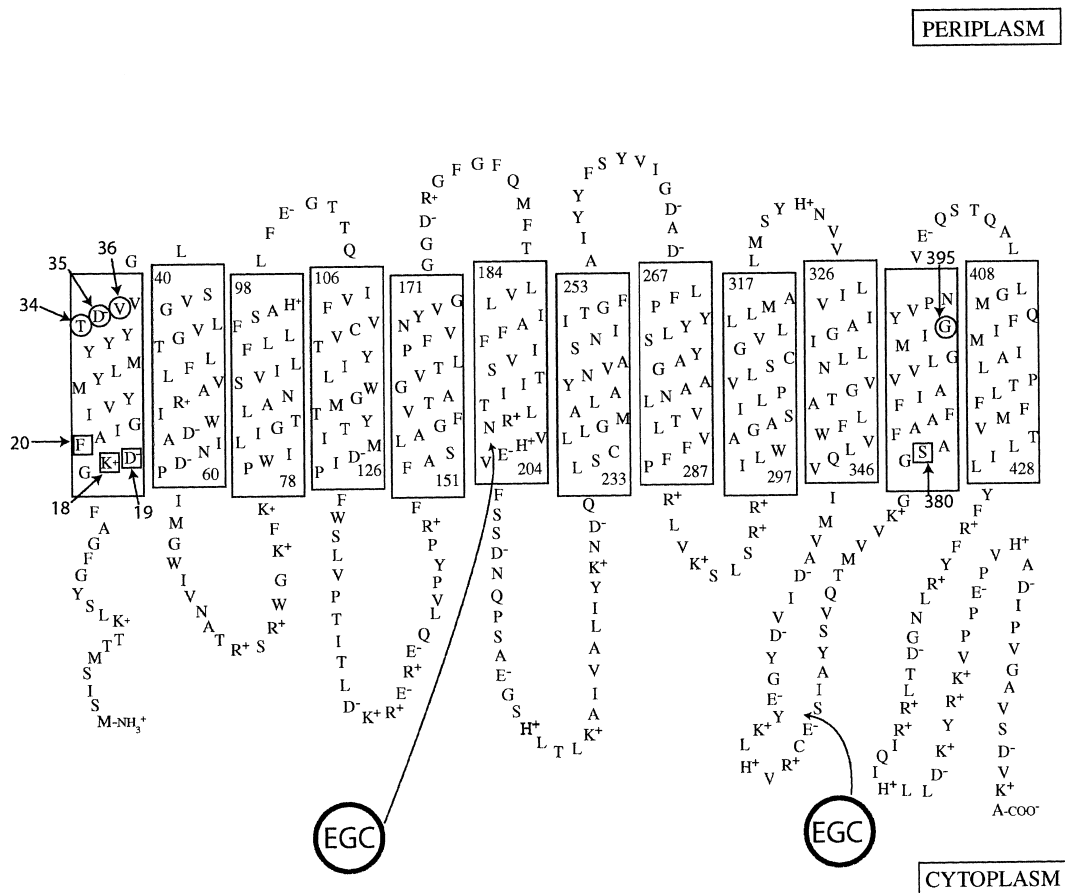


Fig. 1. Secondary structure of the melibiose carrier protein of *E. coli*, with 12 transmembrane helices connected by loops and both N- and C-termini on the cytoplasmic side (adapted from [24]). Circled residues are sites for double cysteine mutations near the periplasmic side; squared residues are sites for double cysteine mutations near the cytoplasmic side. EGC indicates the two cleavage sites (203E and 365E) by endoproteinase Glu-C [12].

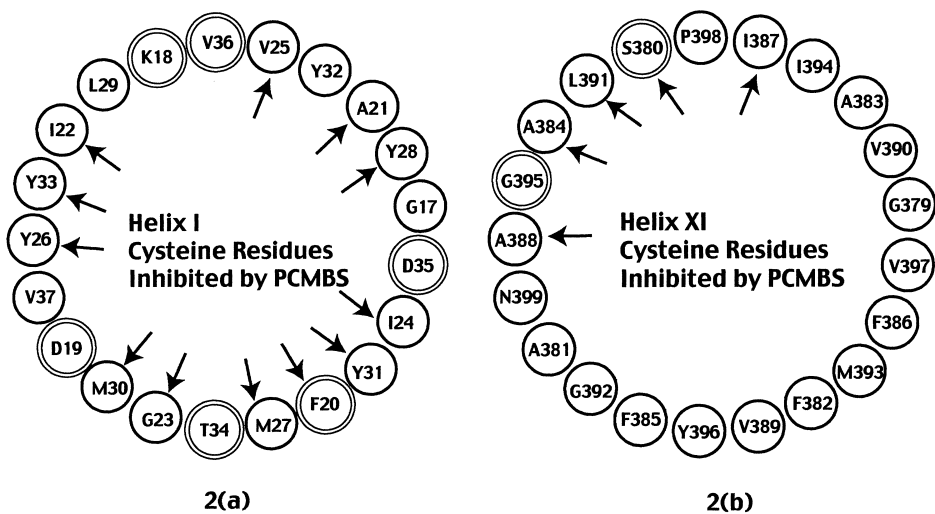


Fig. 2. Helical wheel plots for helix I [20] and helix XI [22]. The arrows indicate sites of PCMBs inhibitions of cysteine mutants. Those sites comprise a face of the helix that faces the aqueous channel. Double-circled residues are sites of cysteine mutations in the double cysteine mutants. As indicated, all the cysteine mutations used in this investigation are facing the aqueous channel.

Table 1

Characteristics of single cysteine mutants relevant to the double mutants used for cross-linking

Helix I ^a			Helix XI ^b		
Mutant	Protein level (%) ^c	Transport (%) ^b	Mutant	Protein level (%) ^c	Transport (%) ^d
K18C	24	0	S380C	22	60
D19C	31	0			
F20C	34	70			
T34C	84	2.5	G395C	44	40
D35C	137	0			
V36C	89	72			

^aData comes from [20].^bData comes from [22].^{c,d}Data are presented as percentages of the C-less parent value.

3.2. Cross-linking

We applied I₂, Cu(Ph)₃, o-PDA and p-PDA to the six double cysteine mutants for cross-linking between helices I and XI. Due to their low levels of carrier protein expression, K18C/S380C and T34C/G395C were not useful for our experiments (unpublished observations). For reasons not clear to us, p-PDM was unsatisfactory (unpublished observations).

We used Endoproteinase Glu-C (EGC) to cleave the melB protein, in order to distinguish cross-linked species from uncross-linked ones. EGC is a highly specific protease that hydrolyzes the peptide bond at the carboxylic side of Glu in ammonium bicarbonate at pH 7.8. According to Gwizdek et al. [12], this enzyme has two cleavage sites in membrane-bound melB carrier protein in inside-out membrane vesicles: 203E (at the bottom of helix VI) and 365E (in loop X/XI) (Fig. 1). C-Terminal antibody detects two

fragments of M_r of 18 and 30 kDa on an 18% SDS–PAGE gel. If helices I and XI were cross-linked, the 18 kDa fragment would not result from the EGC cleavage. Instead, there would be a band from two pieces of the melB protein joined together by cross-linking, with a M_r about 30 kDa (the entire melB protein minus the fragment between 203E and 365E). This band would overlap with the 30 kDa band from the EGC-treated melB on the gel. The intensity of the 18 kDa band on the gel would be a valid diagnostic tool to detect cross-linking, using C-terminal antibody of melB. The results of the cross-linking experiments are in Fig. 3.

3.2.1. D19C/S380C

We used I₂ and Cu(Ph)₃ to attempt to cross-link the two cysteine residues, but the experiment was not successful. After EGC cleavage the 30 and 18 kDa bands were essentially the same for the control as for

Table 2

Characterization of double cysteine mutants for cross-linking

Mutant	Carrier protein expression level (%) ^a	Transport activity (%) ^b	Both cysteines in aqueous channel? ^c
K18C/S380C	5 ± 1	0	Yes
D19C/S380C	10 ± 2	0	Yes
F20C/S380C	70 ± 8	90	Yes
T34C/G395C	4 ± 1	0	Yes
D35C/G395C	37 ± 4	0	Yes
V36C/G395C	15 ± 2	14	Yes

^aThe protein expression level is presented as a percentage of that of the C-less parent.^bThe transport activity is presented as a percentage of that of the C-less parent. Melibiose was 0.1 mM, Na⁺ was 10 mM, accumulation for 10 min.^cThe orientation of the cysteine residues was judged on the basis of PCMBs inhibition pattern of the helical wheel plot of the helix (Fig. 2).

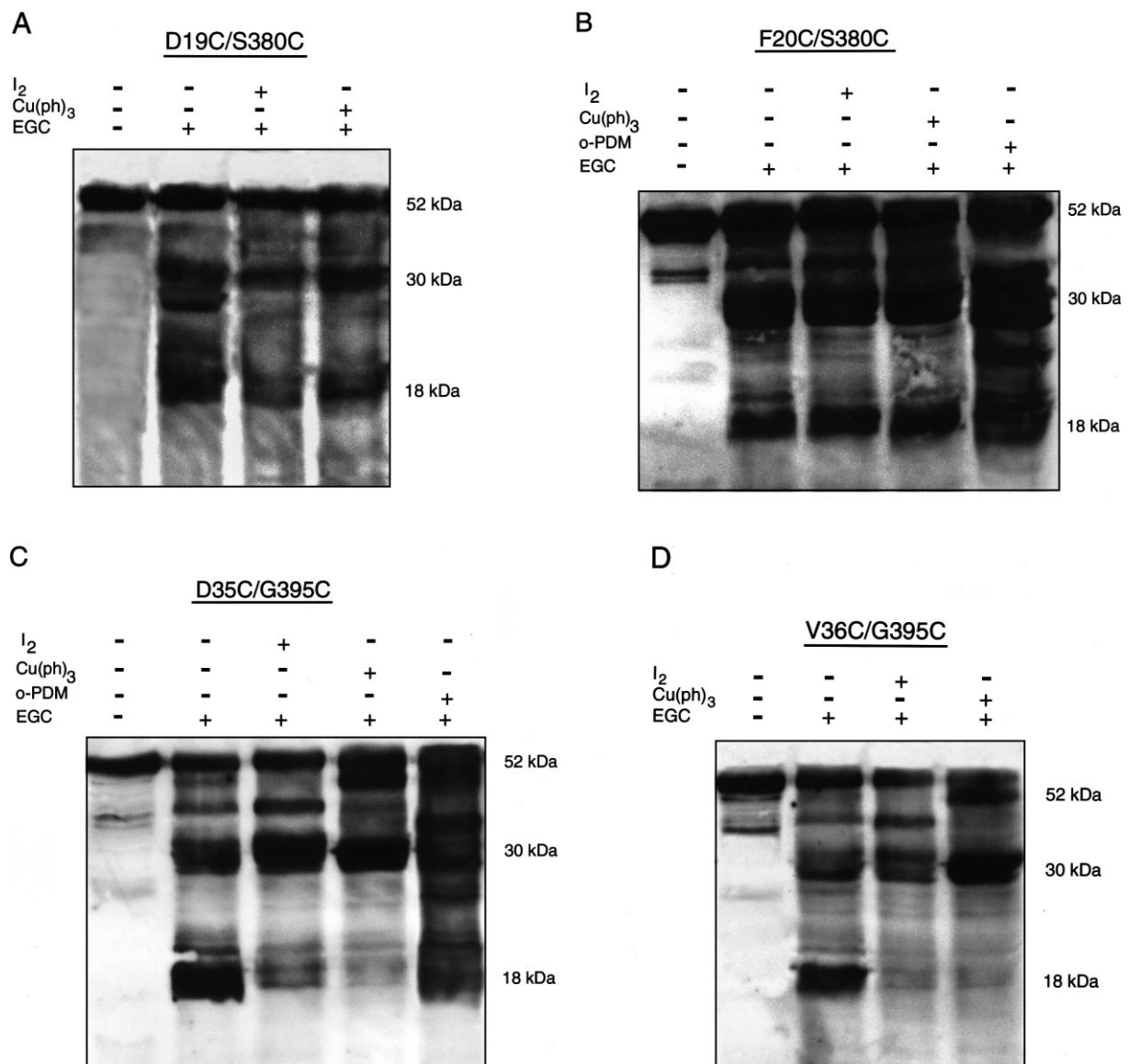


Fig. 3. Endoproteinase Glu-C (EGC) cleavages of cross-linking assay samples as shown on 18% SDS-PAGE gel. Detection of the bands was by C-terminal antibody to melB protein visualized by chemiluminescence (see Section 2). (A) Result of D19C/S380C, showing little or no cross-linking, as indicated by the similar intensities of the 18 kDa bands (explained in Section 3). (B) Result of F20C/S380C, showing little or no cross-linking, indicated by the similar intensities of the 18 kDa bands. (C) Result of D35C/G395C, showing significant cross-linking by I₂ and Cu(Ph)₃ but not o-PDM, indicated by the differences in the intensities of the 18 kDa bands. (D) Result of V36C/G395C, showing significant cross-linking by I₂ and Cu(Ph)₃, indicated by the differences in the intensities of the 18 kDa bands.

the cross-linking assays (Fig. 3A). We also tried o-PDM, but the results were not useful (unpublished observation).

3.2.2. F20C/S380C

We tried I₂, Cu(Ph)₃ and o-PDM to attempt to cross-link the two residues, but was not successful

with any of the three reagents. As shown in Fig. 3B, the 30 and 18 kDa bands were essentially the same for the control as for the cross-linking assays.

3.2.3. D35C/G395C

We attempted to cross-link the two cysteine residues by using I₂, Cu(Ph)₃ and o-PDM. While the

o-PDM assay looked essentially the same as the control with respect to the 30 and 18 kDa bands, the I_2 and $Cu(Ph)_3$ assays showed a much lighter band of 18 kDa than the control (Fig. 3C), indicating significant cross-linking. We interpret this as the immediate proximity between C35 and C395.

3.2.4. V36C/G395C

We applied I_2 and $Cu(Ph)_3$ for cross-linking. Both assays showed a much lighter 18 kDa band than the control (Fig. 3D), indicating significant cross-linking between 36C and 395C. We interpret this as the immediate proximity between the two residues. We also tried o-PDM, but for some unknown reason the result was not useful (unpublished observations).

4. Discussion

In the absence of the crystal structures, a combination of site-directed mutagenesis and screening of mutants (e.g., with altered sugar and cation recognition, and second-site revertants) can provide useful information regarding the structure and function relation of the membrane-bound melibiose carrier and other membrane proteins. Proximities between the transmembrane helices are an important feature of the protein structure. This can be directly estimated by applying a variety of cross-linking reagents of different lengths to different locations on helices in question (at cytoplasmic or periplasmic ends, or in the middle of the helix). This has been demonstrated in lactose permease [32,33]. The same technique has also been used to estimate the proximity of loops [34,35] and conformational changes upon the binding of the substrate [35,36] in lactose permease.

In this study we demonstrated that in the absence of Na^+ and melibiose, the membrane bound carrier protein had its helices I and XI in immediate proximity to each other at the periplasmic end, though not at the cytoplasmic end. The choice of the two helices was based on our previous studies on second-site revertants [25] which suggested an interaction or proximity between the two helices. The thiol cross-linking result is a confirmation and refinement of the physiological evidence. Through this experiment we wish to illustrate the validity of second-site revertants screening in combination with chemical cross-

linking to elucidate structural features of membrane-bound proteins.

We used protease EGC to cleave the membrane-bound carrier protein in inside-out vesicles so that the cleavage pattern would distinguish cross-linked molecules from those uncross-linked. When the non-cross-linked normal protein is cut by the proteolytic enzyme it resulted in three bands identified with an antibody to the C-terminal 10 amino acids: 52 kDa (uncleaved protein), 30 kDa (EGC cuts once at 203E) and 18 kDa (EGC cuts at both 203E and 365E). Cross-linking between helix I and helix XI results in binding of the 18 kDa fragment to the N-terminal fragment. This joint fragment is about 30 kDa. Certain proteins, such as metal-tetracycline/ H^+ antiporter [37], when cross-linked within the molecule, have a markedly different mobility on SDS-PAGE gel from that of the uncross-linked, presumably due to the two very different configurations before and after cross-linking. This makes a convenient detection without dependency on proteases. In our case, we have not detected any perceptible changes in the apparent molecular mass of the protein when treated with cross-linking reagents (unpublished observation). Our method of using protease as the analysis tool is affected by the cleavage pattern (two cleavage sites are better than one site for the sake of detection), and the enzymatic efficiency. The method unfortunately cannot detect very low extent of cross-linking for this would make little difference on the result of the cleavage as visualized with immunoblot. Another disadvantage of the protease method is its susceptibility to inhibition by cation and substrate of the carrier protein [12]. This was interpreted as that the conformational changes upon the binding of the cation and substrate protected the carrier protein from protease cleavage. We found that the presence of melibiose and Na^+ in the cross-linking assay resulted in poor enzymatic activity (unpublished observation), consistent with the observation that cation or cation plus melibiose significantly lowered the efficiencies of proteinase K, thermolysin and trypsin [12]. This unfortunately prevented us from using melibiose together with Na^+ to detect any conformational changes in the carrier protein upon binding. However, we found that the presence of 10 mM Na^+ did not seem to affect our results of F20C/S380C and V36C/G395C cross-link-

ings (unpublished observation), suggesting that the binding of cation does not significantly alter the orientations of helix I and helix XI with respect to each other. We also noticed that there are other potential cleavage sites for EGC in the cytoplasmic loops, in addition to the two sites indicated in Fig. 1. These sites, however, were not obviously used by the EGC under our experimental conditions, consistent with the result from Gwizdek et al. [12]. We speculate that such sites are normally hidden in locations not easily accessible to the enzyme, consistent with the notion that loops are not always of flexible random structure but may have defined local conformations. Yet higher concentrations of EGC seem to have effect on those sites, as judged from results from Gwizdek et al. [12]. Under the circumstances, our enzymatic cleavage of the carrier protein is not complete, but it does not affect our conclusion. In certain cases we seem to get more bands than expected on the blots (even though they do not change our conclusions); we interpret this as various extents of non-specific cleavage of the carrier protein. Finally, we would like to argue that the double cysteine mutants used in our cross-linking experiments approximate the native structure of the carrier protein, even though they are devoid of transport activity. D19C/S380C and D35C/G395C lost their transport activities because D19 and D35 are essential in cation/sugar recognition. It is unlikely that they undergo significant global structural changes.

Acknowledgements

This work was supported by National Institutes of Health Grant DK05736.

References

- [1] G. Leblanc, T. Pourcher, M.L. Zani, in: L. Reuss, J.M. Russell, M.L. Jennings (Eds.), *Molecular Biology and Function of Carrier Protein*, Rockefeller University Press, New York, 1993.
- [2] B. Poolman, J. Knol, C. van der Does, P.J.F. Henderson, W.-J. Liang, G. Lablanc, T. Pourcher, I. Mus-Veteau, *Mol. Microbiol.* 19 (1996) 911–922.
- [3] J. Lopilato, T. Tsuchiya, T.H. Wilson, *J. Bacteriol.* 134 (1978) 147–156.
- [4] T. Tsuchiya, M. Oho, S. Shiota-Niiya, *J. Biol. Chem.* 258 (1983) 12765–12767.
- [5] T. Tsuchiya, J. Raven, T.H. Wilson, *Biochem. Biophys. Res. Commun.* 76 (1977) 26–31.
- [6] T. Tsuchiya, T.H. Wilson, *Membr. Biochem.* 2 (1978) 63–79.
- [7] M. Hanatani, H. Yazyu, S. Shiota-Niiya, Y. Moriyama, H. Kanazawa, M. Futai, T. Tsuchiya, *J. Biol. Chem.* 259 (1984) 1807–1812.
- [8] H. Yazyu, S. Shiota-Niiya, T. Shimamoto, H. Kanazawa, M. Futai, T. Tsuchiya, *J. Biol. Chem.* 259 (1984) 4320–4326.
- [9] M.C. Botfield, K. Naguchi, T. Tsuchiya, T.H. Wilson, *J. Biol. Chem.* 267 (1992) 1818–1822.
- [10] T. Pourcher, S. Leclercq, G. Grandolin, G. Leblanc, *Biochemistry* 34 (1995) 4412–4426.
- [11] T. Pourcher, E. Bibi, H.R. Kaback, G. Leblanc, *Biochemistry* 35 (1996) 4161–4168.
- [12] C. Gwizdek, G. Leblanc, M. Bassilana, *Biochemistry* 36 (1997) 8522–8529.
- [13] D.E. Cohn, H.R. Kaback, *Biochemistry* 19 (1980) 4237–4243.
- [14] E. Damiano-Forano, M. Bassilana, G. Leblanc, *J. Biol. Chem.* 261 (1986) 6893–6899.
- [15] M. Bassilana, E. Damiano-Forano, G. Leblanc, *Biochem. Biophys. Res. Commun.* 129 (1985) 626–631.
- [16] T. Pourcher, M.L. Zani, G. Leblanc, *J. Biol. Chem.* 268 (1993) 3209–3215.
- [17] M.L. Zani, T. Pourcher, G. Leblanc, *J. Biol. Chem.* 268 (1993) 3216–3221.
- [18] M.L. Zani, T. Pourcher, G. Leblanc, *J. Biol. Chem.* 269 (1994) 24883–24889.
- [19] D.M. Wilson, T.H. Wilson, *Biochim. Biophys. Acta* 1190 (1994) 225–230.
- [20] P.Z. Ding, T.H. Wilson, *Biochemistry* 40 (2001) 5506–5510.
- [21] S. Matsuzaki, A.C. Weissborn, E. Tamai, T. Tsuchiya, T.H. Wilson, *Biochim. Biophys. Acta* 1420 (1999) 63–72.
- [22] P.Z. Ding, T.H. Wilson, *J. Membr. Biol.* 174 (2000) 135–140.
- [23] P.Z. Ding, A.C. Weissborn, T.H. Wilson, *J. Membr. Biol.* (2001) submitted.
- [24] P.J. Franco, T.H. Wilson, *J. Bacteriol.* 181 (1999) 6377–6386.
- [25] P.Z. Ding, T.H. Wilson, *Biochem. Biophys. Res. Commun.* 268 (2000) 409–413.
- [26] P.J. Franco, J.B. Anupam, T.H. Wilson, *Biochim. Biophys. Acta* 1510 (2001) 231–242.
- [27] D.M. Wilson, T.H. Wilson, *Biochim. Biophys. Acta* 904 (1987) 191–200.
- [28] A.C. Weissborn, M.C. Botfield, K. Masayuki, T. Tsuchiya, T.H. Wilson, *Biochim. Biophys. Acta* 1329 (1997) 237–244.
- [29] S. Lolkema, I.B. Puttner, H.R. Kaback, *Biochemistry* 27 (1988) 8307–8310.
- [30] M.C. Botfield, T.H. Wilson, *J. Biol. Chem.* 264 (1989) 11649–11652.
- [31] B. Rosen, T. Tsuchiya, *Methods Enzymol.* 56 (1979) 233–241.

- [32] J. Wu, D. Hardy, H.R. Kaback, *Biochemistry* 38 (1999) 1715–1720.
- [33] Q. Wang, H.R. Kaback, *Biochemistry* 38 (1999) 16777–16782.
- [34] J. Sun, H.R. Kaback, *Biochemistry* 36 (1997) 11959–11965.
- [35] I. Kwaw, J. Sun, H.R. Kaback, *Biochemistry* 39 (2000) 3134–3140.
- [36] J. Wu, D. Hardy, H.R. Kaback, *J. Mol. Biol.* 282 (1998) 959–967.
- [37] Y. Kubo, S. Konishi, T. Kawabe, S. Nada, A. Yamaguchi, *J. Biol. Chem.* 275 (2000) 5270–5274.